Title

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Journal

Journal of perinatology : official journal of the California Perinatal Association, 33(9)

ISSN

0743-8346

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Publication Date

2013-09-01

DOI

10.1038/jp.2013.41

Peer reviewed

HHS Public Access

Author manuscript

J Perinatol. Author manuscript; available in PMC 2014 March 01.

Published in final edited form as:

J Perinatol. 2013 September ; 33(9): 691–697. doi:10.1038/jp.2013.41.

Probiotic Administration in Congenital Heart Disease: A Pilot Study

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Abstract

Objective—Investigate the impact of probiotic *Bifidobacterium longum* ssp *infantis* on the fecal microbiota and plasma cytokines in neonates with congenital heart disease.

Study design—Sixteen infants with congenital heart disease were randomly assigned to receive either *B. infantis* (4.2×10^9 cfu twice daily) or placebo for 8 weeks. Stool specimens from enrolled infants and from six term infants without heart disease were analyzed for microbial composition. Plasma cytokines were analyzed weekly in the infants with heart disease.

Results—Healthy control infants had increased total bacteria, total *Bacteroidetes*, and total bifidobacteria compared to the infants with heart disease, but there were no significant differences between the placebo and probiotic groups. Plasma IL10, IFNγ, and IL1β levels were transiently higher in the probiotic group.

Conclusions—Congenital heart disease in infants is associated with dysbiosis. Probiotic *B. infantis* did not significantly alter the fecal microbiota. Alterations in plasma cytokines were inconsistent.

Keywords

probiotic; bifidobacteria; necrotizing enterocolitis; congenital heart disease; microbiota

Introduction

Necrotizing enterocolitis (NEC) is predominantly a disease of premature infants. Among term infants, the prevalence of NEC has been estimated at 1 in 20,000 live births.[1] Congenital heart disease (CHD) is one of the most consistent risk factors for NEC in term

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Conflict of Interest: None of the authors has a conflict of interest.

infants. In two case series about one third of term infants with NEC had CHD.[1, 2] Among term infants with CHD requiring surgery in the neonatal period, the incidence of NEC has been reported to be 3–7%, which is similar to the incidence among premature infants.[3, 4] Mortality rates among infants with CCHD who develop NEC, however, may be even greater than in premature infants[5, 6] with one study reporting mortality as high as 71%.[7]

In premature infants primary risk factors for NEC include immaturity, enteral feeding, and atypical development of the intestinal microbiota (dysbiosis).[8] In premature infants the most promising interventions in prevention of NEC include human milk[9] and probiotics.[10] Probiotics have been shown to decrease gene expression of pro-inflammatory cytokines[11] and to alter the intestinal microbiota.[12] Whether the pathophysiology of NEC in term infants with CHD is similar to that in premature infants is unknown.

We performed a pilot study of probiotic administration to term infants scheduled for cardiac surgery within the first weeks of life. Our hypotheses were that term infants with CHD would have a markedly different intestinal microbiota than healthy term infants and that administration of probiotic bifidobacteria to term infants with CHD would alter the intestinal microbiota and the levels of plasma cytokines.

Patients and Methods

This trial was performed at UC Davis Children's Hospital in Sacramento, California from Aug 2009 to Dec 2010 following approval from the Institutional Review Board and registration at clinicaltrials.gov (NCT01018472). Eligible infants included those with gestational age at least 34 weeks, congenital heart disease requiring repair in the neonatal period, and absence of congenital gastrointestinal anomalies. Following informed consent from the parents, infants were randomly assigned by the UC Davis Investigational Pharmacy to receive either 4.2×10^9 colony forming units of *Bifidobacterium longum* ssp *infantis* ATCC 15697 or a placebo twice daily for eight weeks or until death or discharge if sooner.

The probiotic strain was chosen based on its genetic capacity to digest human milk oligosaccharides, an advantage in colonizing the neonatal intestinal tract.[13] To avoid the drawbacks of over-the-counter probiotic products (e.g. inconsistent composition and viability)[14] the probiotic strain was grown by a food-grade commercial facility (Culture Systems, Inc., Mishawaka, IN) and stored at −80 degrees C. Purity and number of viable bacteria was confirmed every six months by culture techniques. The placebo was a dilute formulation of Pregestimil powder (Mead Johnson) without significant caloric value. The investigators, clinical research coordinator, caregivers and parents were blinded as to group of assignment.

Feces were obtained from a soiled diaper prior to the first dose of the study product and then weekly thereafter when possible. Fecal samples were refrigerated overnight, transported on dry ice and stored at −80 degrees C. One ml of blood was obtained at enrollment and then weekly for 5 weeks when possible. The blood was centrifuged at 3000 rpm for 15 minutes and the plasma frozen at −80 degrees C.

The healthy comparison group consisted of 6 term breast-fed infants. None had congenital heart disease, required hospitalization or were treated with antibiotics or probiotics during the study period. Stool samples were obtained from a soiled diaper by the parents at regular intervals and kept on ice until transport within 12 hours and storage at −80 degrees C.

DNA extraction

Bacterial genomic DNA was extracted from stool samples as previously described with a few modifications.[15] Briefly, frozen stool was thawed and centrifuged (8,000 × g for 5 min at room temperature). The fecal pellet was rinsed twice with PBS then resuspended in 200 μl lysis buffer (2 mM EDTA, 1.2 % TritonX-100, 20 mM Tris-HCl, pH 8.0) with freshly added 40 mg/ml lysozyme. The solution was incubated at 37°C for 30 min. Buffer ASL from QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) was added to equal 2.0 ml, vortexed until thoroughly mixed, and then homogenized by bead-beating in a FastPrep-24 Instrument (MP Biomedicals, Solon, OH) for 2 min at 6.5 m/sec. The homogenate was incubated for 5 min at 95°C, vortexed, and centrifuged at 13,000 × g for 1 min to pellet stool particles. DNA in the supernatant was purified with the Qiagen Stool Mini Kit.

qPCR

SYBR green and TaqMan quantitative real time PCR (qPCR) assays were performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA) with primers specific for universal eubacteria and bifidobacteria. Universal eubacteria SYBR green assays contained 10 μl of 2x Takara Perfect Real Time master mix (Clontech Laboratories, Mountain View, CA), 6 μl water, 400 nM of forward and reverse primers (all primers listed in the supplementary information Table A), and 2 μl diluted 1:100 genomic DNA with cycling conditions as previously described.[16] Bifidobacteria TaqMan qPCR assays contained 12.5 μl 2x TaqMan Universal PCR master mix (Applied Biosystems), 300 nM of forward and reverse primers, 150 nM TaqMan probe, 3.75 μl water, and 2.5 μl diluted 1:100 genomic DNA[17] with cycling conditions as previously described.[16] All reactions were carried out in triplicate with a nontemplate control.

TRFLP analysis of the fecal microbiota

PCR amplification was performed in 50-μL reactions containing 1–5 ng of DNA template, 25 μL 2X Promega GoTaq Green Master Mix (Promega, Madison, WI), 1 mM $MgCl_2$, and 2 pmol of each primer). [18,19] Each PCR was performed in triplicate and the products combined prior to purification. The PCR conditions were an initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 2 min, with a final extension at 72°C for 5 min.

A focused TRFLP of non-clostridial *Firmicutes* was performed as described.[20] PCR conditions consisted of an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 45 sec, annealing at 66°C for 30 sec, and extension at 72°C for 45 sec, with a final extension at 72°C for 5 min.

PCR products were analyzed by electrophoresis and purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). All 16S-TRFLP amplicons were digested using the

enzymes AluI, MspI, HaeIII, and HhaI. Focused TRFLP amplicons were digested using the enzymes MseI, Hpy188I, and Hpy188III. Next, 1.5 μl of the digestion mixture was used for fragment analysis and traces were visualized with the program Peak Scanner v1.0 (Applied Biosystems). Peak filtration and clustering analyses were performed with R software, using published program scripts and analysis protocols.[21]

Taxonomic assignments of operational taxonomic units were based on comparison to an *in silico* digest database generated by the virtual digest tool from MiCA[22] of good-quality 16S rRNA gene sequences compiled by the Ribosomal Database Project Release.[23, 24]

Cytokine analysis

Plasma cytokines were quantified using high sensitivity human multiplexing bead immunoassays (Millipore Corporation, St. Charles, MO) based on a sandwich immunoassay utilizing the Luminex fluorescent-bead-based technology following established protocols[25]. Briefly, 50 ul of plasma were incubated with antibody-coupled beads. After a series of washes, a biotinylated detection antibody was added followed by streptavidin-phycoerythrin. The bead sets were analyzed using a flow-based Luminex™ 100 suspension array system (Bio-Plex 200; Bio-Rad Laboratories, Inc.). Unknown sample cytokine concentrations were calculated by Bio-Plex Manager software using a standard curve derived from the known reference cytokine concentrations. A five-parameter model was used to calculate final concentrations.

Statistical analysis

Using QIIME software, multidimensional summary measures of the microbial composition of the fecal samples analyzed by 16S-TRFLP were derived and visualized in a reduced number of dimensions via a 3-dimensional principal coordinate analysis (PCoA) plot.[26] Shannon diversity indices were calculated from the TRFLP data.

qPCR and cytokine data were log transformed and analyzed in STATA (version 9, 2007) using repeated measures ANOVA. For data that did not approximate a normal distribution after log transformation, the non-parametric Kruskal-Wallis test was used at specific time points. For the baseline and combined qPCR data and the combined Shannon diversity indices standard one-way ANOVA with Scheffe's test for differences between groups was used. For the qPCR data, readings of zero were presumed to represent 1×10^4, the lower limit of detection. Given that this pilot study was designed to generate hypotheses rather than test them we report p values < 0.1 as being of interest for possible future testing.

Results

Sixteen infants with congenital heart disease were enrolled in the study. Two of the infants were removed from the study prior to receiving the study product, one because of transfer to another facility (placebo group) and one at the request of the parents (probiotic group). Baseline specimens, demographic and clinical data were included for both on an intention-to-treat basis. A summary of patient details is provided in Table 1 with patient-specific details in the supplementary information (Table B). The groups were similar except for age at enrollment.

TRFLP and qPCR are complementary techniques with the former providing an overview of all identifiable bacteria and the latter providing more precise quantification of total bacteria and specific taxa. Figure 1 presents the mean percentages of bacterial phyla by TRFLP for each group over time. *Bacteroidetes* were more common in the healthy control infants than the CHD infants at baseline, weeks 1–6, and weeks 7 and greater (Kruskal-Wallis p=0.036, 068, and 0.041). *Actinobacteria* showed the same pattern (Kruskal-Wallis p=0.017, 0.023, and 0.035 at the same time points). *Firmicutes* were more common in infants with CHD but only at the middle time points (weeks 1–6, Kruskal-Wallis p=0.052). No significant differences were noted between the placebo and probiotic groups. The TRFLP data for each individual infant at the class/order level are presented in the supplementary information (Figure A). The phylum *Firmicutes* includes the Gram-positive classes *Clostridia, Bacilli*, and *Negativicutes*. To characterize the non-clostridial *Firmicutes*, focused TFRLP analysis was performed for each specimen for three of the control infants and fourteen of the infants with heart disease. All groups showed a predominance of streptococci with no detectable lactobacilli. A trend towards decreased enterococci in the healthy controls did not reach statistical significance (supplementary information: group comparison in Figure B with individual analyses in Table C. Figure 2 is the principal coordinate analysis of the TRFLP data. Each circle represents one specimen with increased distance between circles reflective of greater differences in microbial composition. The Shannon diversity indices [mean(SD)] were 2.3(0.22) for the infants without CHD, 2.1(0.40) for the probiotic group, and 1.92(0.51) for the placebo (ANOVA p=0.24).

Figure 3 presents the mean qPCR data for total bacteria, total *Enterobacteriaceae*, and total bifidobacteria for each group over time. The healthy control infants without CHD had greater total bacteria, total *Enterobacteriaceae*, and total bifidobacteria at baseline and in the combined non-baseline specimens (Figure 4). The individual q-PCR analyses for each infant are presented in the supplementary information (Figure C). Primers specific for *B. longum* were used to assess what proportion of fecal bifidobacteria are the same species as the ingested probiotic. Among the infants receiving the probiotic, 93% of the bifidobacteria identified were *B. longum*, whereas among the infants receiving placebo, 72% were *B. longum*. Whether this represents cross-contamination as has been demonstrated in other probiotic studies[27] is unclear. The mean percentage of bifidobacteria identified as *B. longum* from three of the control infants (B, C, and D) were 43%, 58%, and 0%.

Figure 4 presents levels of four cytokines in the CHD infants. Differences between groups over time were significant for IFNγ (p=0.007) and IL1β (p=0.04), with the following differences at individual weeks: IFNγ week 1 (p=0.08), IL1β week 3 (p=0.06), and IL10 week 2 (p=0.06). No trends were noted for TNFα or IL8 (data not shown).

Discussion

In the premature infant, NEC is common and devastating. The pathophysiology of this disease is incompletely understood. Major risk factors in the premature infant include degree of prematurity, formula feeding, and dysbiosis.[8] Other possible risk factors include red blood cell transfusion[28] and maternal smoking.[29] Prematurity remains the most prominent predictor of NEC risk and is likely related to immaturity of multiple aspects of gastro-

intestinal mucosal immunity (including barrier integrity, signaling, pro-inflammatory responses, Paneth cell function, secretion of antimicrobial molecules, autophagy, and apoptosis).[30–32] Four strands of evidence link dysbiosis and NEC in the premature infant. First, prolonged antibiotic exposure is associated with NEC.[33] Second, increased numbers of Gram-negative *Enterobactericeae* dominate the feces of NEC patients.[34, 35] Third, multiple clinical trials demonstrate decreased risk of NEC in premature infants receiving probiotics.[36] Fourth, acid suppression changes the fecal microbiota and increases risk of developing NEC in premature infants.[37, 38]

In the term infant, NEC is relatively rare. Among this population, the association between CHD and NEC is well established, however given the relatively small number of cases of NEC in term infants with heart disease at any single institution, the pathophysiology of this form of NEC is unexplored.[39] In particular it is unknown whether formula feeding, dysbiosis, transfusion, maternal smoking, dysfunction of mucosal immunity, or alterations in oxygenation or perfusion of the intestine are significant risk factors in this population.

The purpose of this pilot study was to generate preliminary data to begin to address two hypotheses: that infants with CHD would demonstrate significant intestinal dysbiosis and that probiotic *B. infantis* would partially “normalize” the microbiota (i.e. shift the composition towards that of the healthy infant). This study supports the first hypothesis in that infants with CHD develop an altered fecal microbiota most marked by decreased total bacteria, decreased *Actinobacteria* (predominantly bifidobacteria), decreased *Bacteroidetes*, decreased *Enterobactericeae*, and increased *Firmicutes* (predominantly enterococci). However, no significant differences in the composition of the fecal microbiota were noted between the two groups of infants with heart disease, suggesting against our second hypothesis. The lack of a significant increase in fecal bifidobacteria with probiotic treatment in this population differs from observations with this organism in hospitalized premature infants (manuscript submitted). It is possible that other influences on the intestinal microbiota including timing and type of enteral feeding, antibiotic treatment, inhibition of acid suppression, and hypoxia and/or ischemia play such a large role in shaping the microbiota that the influence of the probiotic is minimal.

Several Gram-negative *Enterobacteriaceae* have been shown to trigger an inflammatory response in the intestinal mucosa and then out-compete the commensal organisms in this pro-inflammatory environment.[40] One possible mechanism for the observed anti-inflammatory effects of probiotics is the displacement of this pro-inflammatory population. We included analysis of plasma cytokines to explore the hypothesis that alterations in the microbiota were associated with changes in key cytokines implicated in NEC. As we were unable to observe significant changes in the microbiota related to the probiotic administration, this hypothesis remains untested in this population.

Pilot studies provide preliminary data to determine sample size and/or justify larger definitive studies. NEC occurs at a younger age in term infants than premature infants suggesting that a beneficial effect of probiotics would need to manifest quickly. Based on changes seen after one week of probiotic or placebo in this study, sample size calculations for definitive studies of several possible outcomes are summarized in Table 2. A clinical

trial of probiotics in term infants with congenital heart disease to decrease risk of NEC would require several hundred infants in each arm, however a moderate sized trial to assess short term increases in fecal bifidobacteria or beneficial changes in cytokines appears justifiable.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Support: This study was funded by the Children's Miracle Network and the United States Department of Agriculture. This publication was also made possible by Grant numbers R01 HD059127 from the Eunice Kennedy Shriver National Institute of Child Health and Development (NICHD) and UL1 TR000002 supported by the National Center for Advancing Translational Sciences.

The authors wish to express gratitude to the nurses and families in the Neonatal Intensive Care Unit and the Pediatric Cardiothoracic Intensive Care Unit at the University of California Davis Children's Hospital, to Heather Overman for assistance with specimen processing, and to Judy Van de Water PhD for assistance with the Luminex assays.

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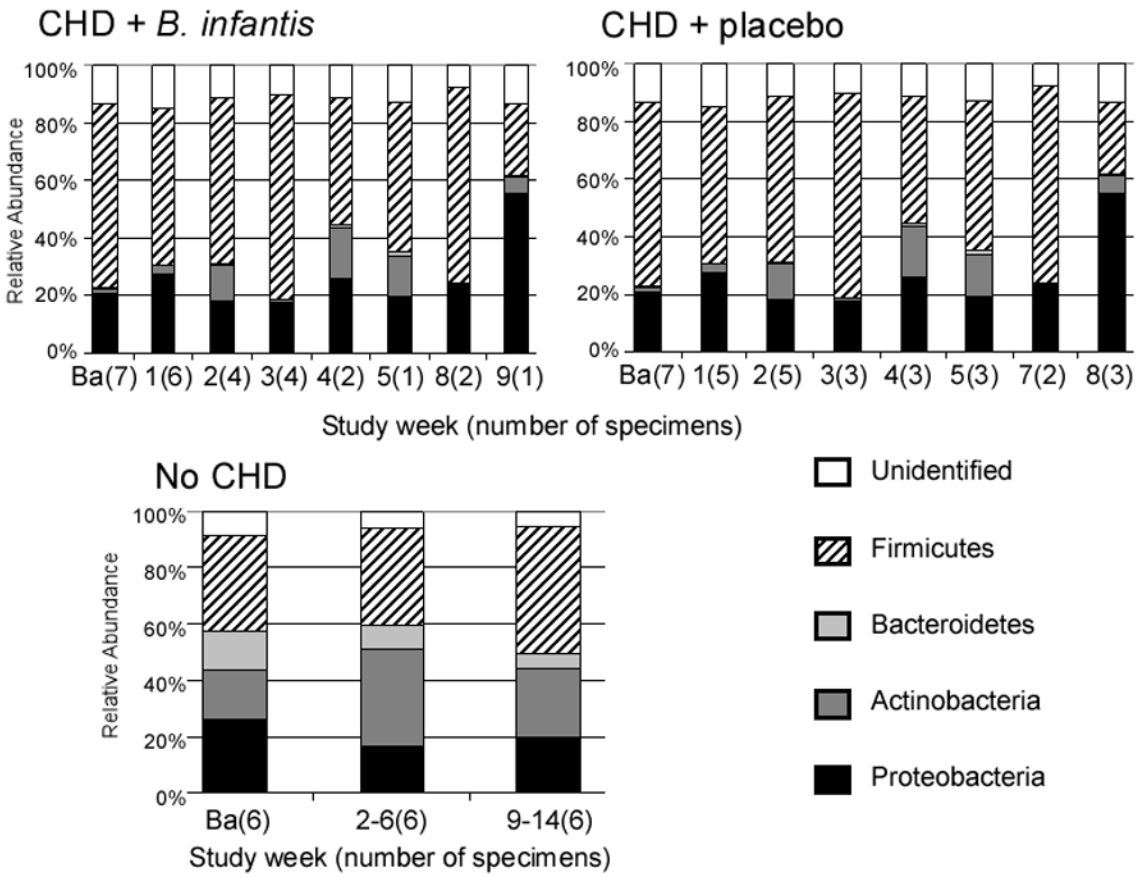

Figure 1.
Mean TRFLP percentages of bacterial phyla by group over time. Ba=baseline specimens.

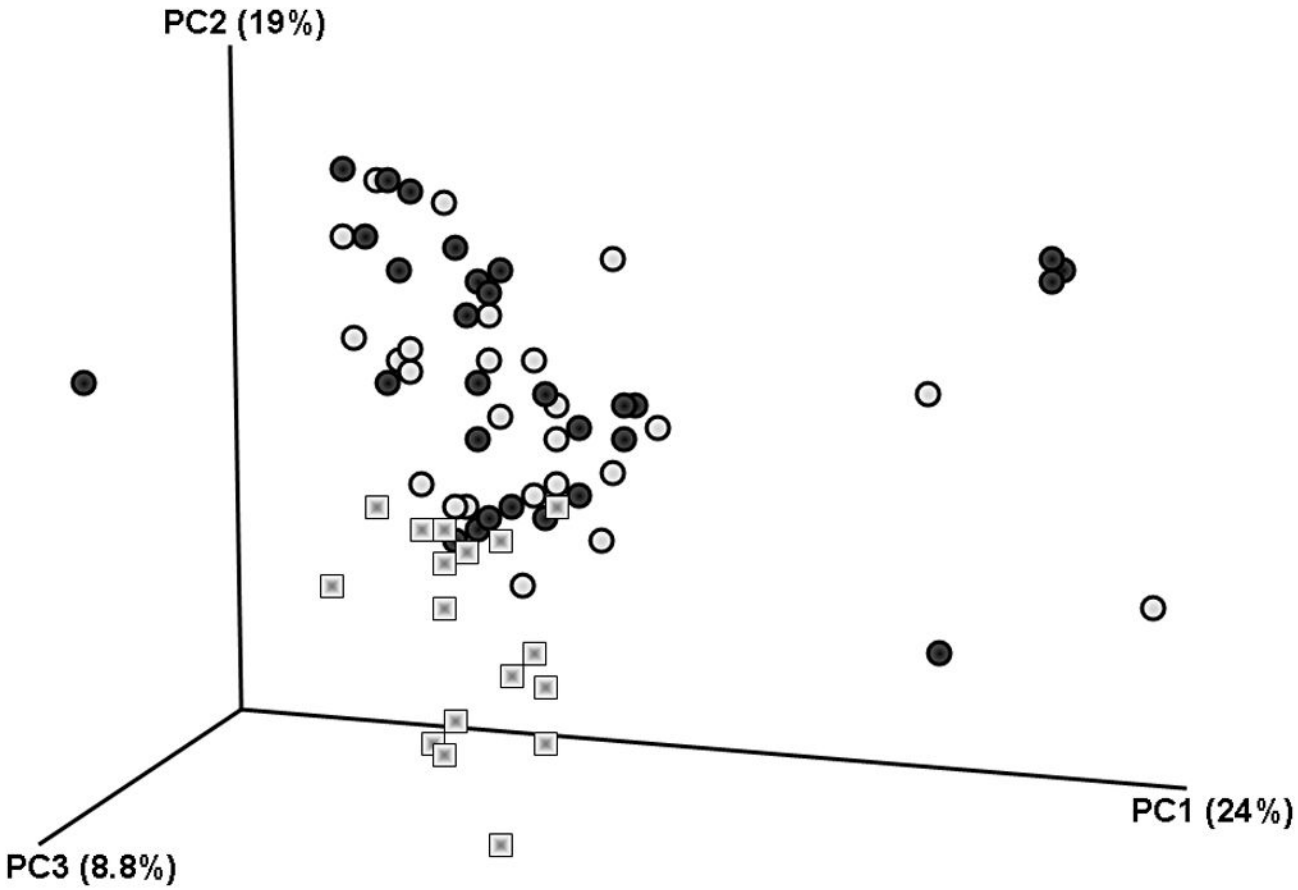

Figure 2.
Principle coordinate analysis of all TRFLP data (open circles = CHD + *B. infantis*, black circles = CHD + placebo, open squares = controls without CHD).

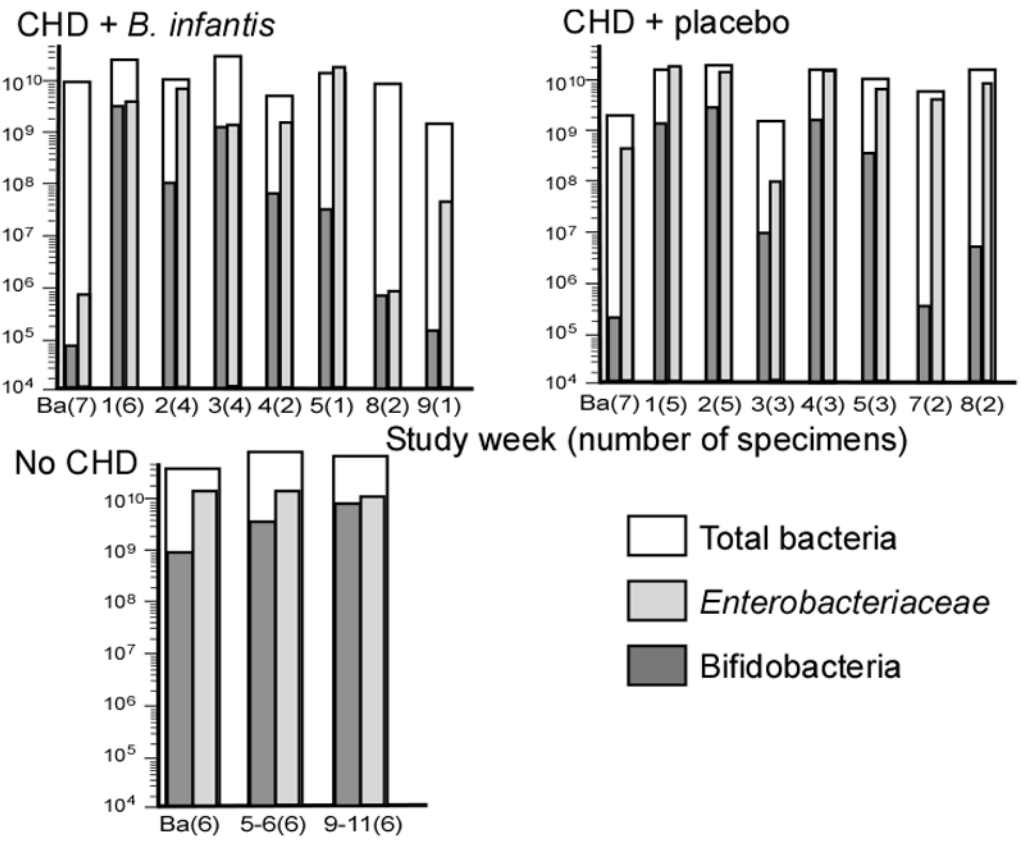

Figure 3.

Mean numbers of total bacteria (white bars), *Enterobacteriaceae* (light gray bars), and bifidobacteria (dark gray bars) by qPCR for each group over time. The Y axis is a logarithmic scale and represents copies of specific 16S rRNA gene sequences (rDNA) per g stool.

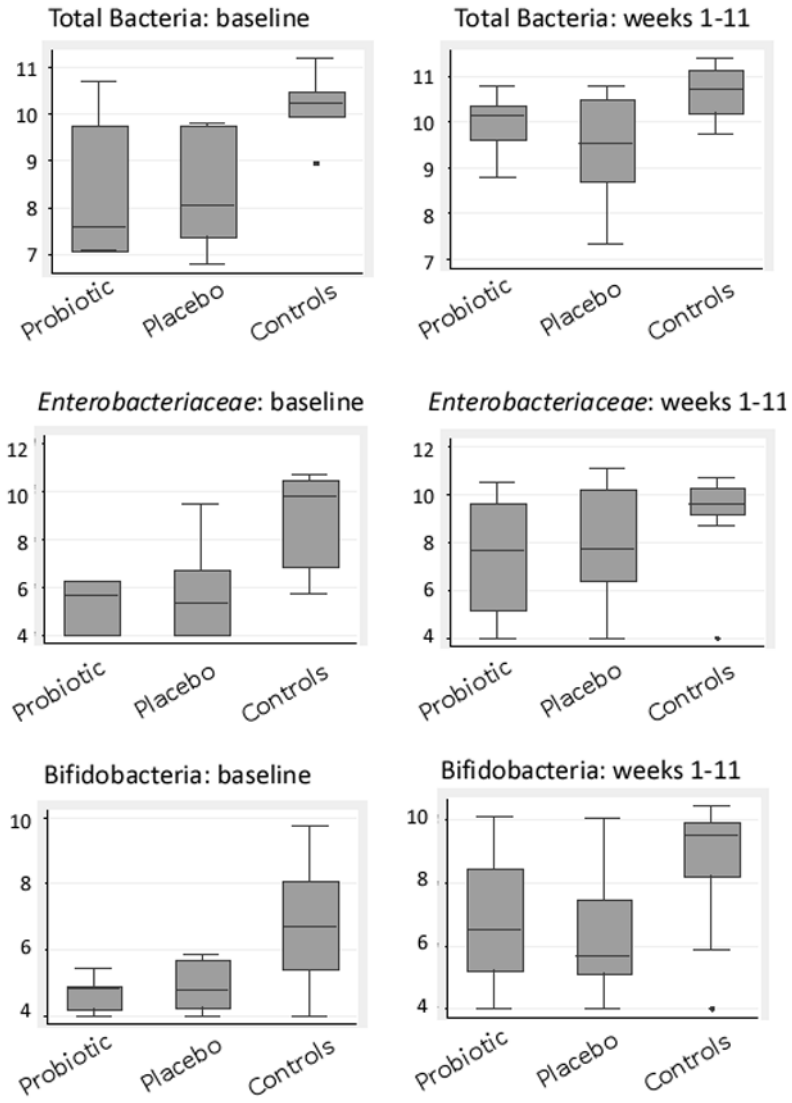

Figure 4.

Box plots of qPCR data for each group at baseline and all subsequent time periods combined. The Y axis is log transformed copies of the 16S rRNA gene per g stool. Total bacteria (baseline ANOVA p=0.03 [Scheffe p=0.07 probiotic v control and p=0.06 placebo v control]; weeks 1–11 ANOVA p=0.001 [Scheffe p=0.002 placebo v control]). Total *Enterobacteriaceae* (baseline ANOVA p=0.003 [Scheffe p=0.006 probiotic v control and p=0.01 placebo v control]; weeks 1–11 ANOVA p=0.07 [Scheffe p=0.07 probiotic v control]). Total bifidobacteria (baseline ANOVA p=0.02 [Scheffe p=0.02 probiotic v control and p=0.05 placebo v control]; weeks 1–11 ANOVA p=0.008 [Scheffe p=0.04 probiotic v control and p=0.01 placebo v control]).

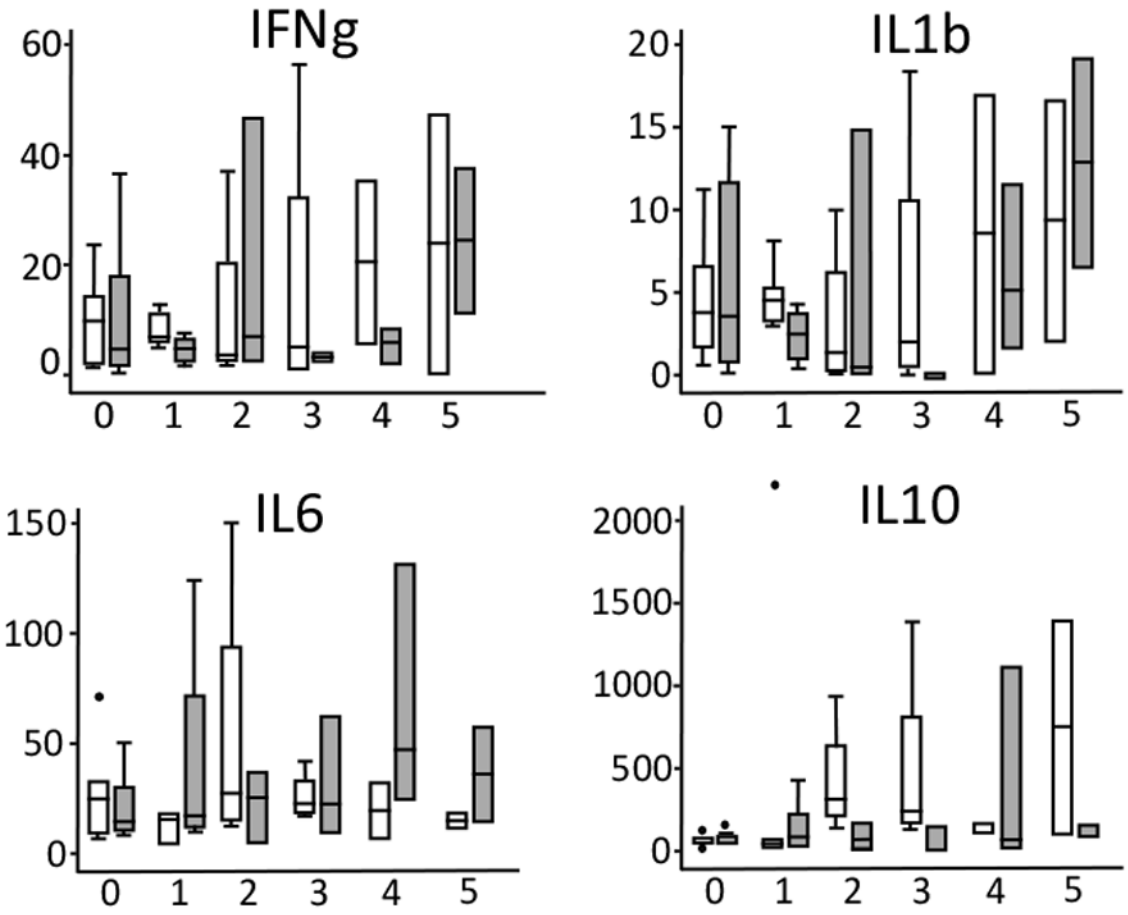

Figure 5.

Box plots of plasma cytokines for the two groups of infants with CHD for each study week. White boxes=probiotic group, gray boxes=placebo group. Y axes are pg/mL.

Table 1

Patient data summary.

	Probiotic (n=8)	Placebo (n=8)
Birth weight gm, mean (SD)	3024 (484)	2889 (533)
Gestational age wks, mean (SD)	38.4 (1.2)	37.4 (2.4)
Delivery (% C-section)	37.5	50
Gender (% male)	37.5	50
1 min Apgar, median (quartiles)	7 (2,8)	8 (7,8)
5 min Apgar, median (quartiles)	8 (8,9)	8 (7,9)
Feeding (% receiving some EBM)	87.5	75
Age at enrollment days, mean (SD)[a]	16.3 (10.4)	7.9 (4.7)
Death	0	1
Necrotizing enterocolitis	0	0
Total days of antibiotics (mean, SD)	34 (42)	56 (69)
Total days NPO, mean (SD)	15 (16)	17 (12)
Day of surgery (mean, SD)	15 (13)	13 (12)
Day of first enteral feeding[b]	14 (12)	7 (5)
Day of full enteral feeding[b]	21 (12)	39 (40)
Total days hospitalized, mean (SD)	53 (43)	80 (86)

[a] p=0.056 (t test).

[b] two infants in the placebo group were not fed enterally during the study period.

Table 2

Sample size calculations based on potentially beneficial observed changes in the first study week (all CHD patients with plasma and blood samples for baseline and week 1).

	Probiotic mean change (SD) n=5	Placebo mean change (SD) n=4	Sample size estimate (per group)
IL6	−4.94 (20.2)	25.8 (56.9)	41
IL8	−10.2 (17.2)	1.94 (15.7)	21
IL10	446 (1010)	86.0 (179)	86
TNFα	−2.42 (9.22)	4.00 (9.09)	43
Total bifidobacteria	4.0×10^9 (5.3×10^9)	5.5×10^6 (1.0×10^7)	19